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Phytochemistry and Antibacterial Activity of *Citrus sinensis* Extracts against Three Pathogenic Bacteria in Benin.

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ABSTRACT

This work is a contribution to the phytochemical and biological studies of *Citrus sinensis* leaves' extracts in Benin. The main chemical groups and total phenols contents of non-volatiles extracts were investigated. Essential oil of *Citrus sinensis* was extracted and analyzed by gas chromatography coupled with mass spectrometry. The antimicrobial activity of various extracts was evaluated on *Staphylococcus aureus*, *Escherichia coli* and *K. pneumoniae* by disk diffusion method. The results obtained showed that the essential oil of *Citrus sinensis* leaves were mainly composed of hydrogenated monoterpens (74%) and oxygenated monoterpens (24%). The major compounds in this oil were sabinene (33.1%) and δ–3-carene (14.9%). Aqueous and semi-ethanolic extracts of this plant were studied and contained anthocyanins, leucoanthocyanes, flavonoids, catechin tannins and saponins. The ethanol extract meanwhile contained leucoanthocyanins, gallic tannins catechin tannins and saponins. Phytochemical studies indicate that the phenolic contents of the non-volatiles extracts varied from 664.00 ± 8.80 to 921.69 ± 0.35 mg EAG / g MS, 31.08 ± 0.11 to 47.78 ± 5.60 mg EC/g MS and 16.98 ± 0.95 to 73.01 ± 2.49 mg EC/g MS respectively for total phenol, flavonoid and tannins. The non-volatiles extracts from *C. sinensis* exhibit no antibacterial activity on *Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae* in contrast to the essential oil that exhibited moderate activity on *S. aureus* and *E coli.*

Keywords: *Citrus sinensis*, *S. aureus*, essential oil, antibacterial activity, pathogenic strains.

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INTRODUCTION

Traditional medicine and the African pharmacopoeia appear more and more as an essential health system for people in developing countries in particular. Its inclusion in the different socio-health development programs is a priority to establish an effective health system. Indeed, its contribution to the treatment of diseases is such that WHO recognizes it as a therapeutic alternative in health systems [1]. Herbal medicine represents one of the most important fields of traditional medicine. Thus, phytotherapy is practiced by a large proportion for the treatment of several physical, physiological and mental problems [2]. To promote the proper uses of herbal medicine and determinate their potential as sources for new drugs it is essential to study medicinal plants, which have folkloric reputation. Within the recent years, infections have increased to a great extent and antibiotics resistance becomes an ever-increasing therapeutic problem [3]. Natural products from plants may give a new source of antimicrobial agents with possibly novel mechanisms of action [4]. *Citrus sinensis* (L.) Osbeck (Navel orange) is a hybrid. This plant contains many medicinally active components from different classes including coumarins, carotenoid, flavonoids [5] and essential oil [6]. Therefore, the present study aimed to describe the chemical composition and antibacterial activities of leaves' extracts of *Citrus sinensis* collected in Benin.

MATERIALS AND METHODS

Plants Material

Leaves of *Citus sinensis* were collected in Cotonou of Benin. They were dried naturally on laboratory benches at room temperature until constant weight. The identification of this plant was made by Professor Yedomonhan of National Herbarium of Benin, University of Abomey-Calavi.

Methods

Phytochemical screening

The plant extracts were screened for the presence of mucilages, alkaloids, flavonoids, steroids and terpenes, anthraquinones, tannins, saponins and heterosides based on the colouring and/or precipitation reactions of the chemical compounds contained in the plant following standards methods. [7, 8]

Alkaloids

Alkaloids were identified by Mayer's reagent and confirmed by Bouchardat test. Formation of cream or brown precipitate respectively indicated the presence of alkaloids [9].

Flavonoids

A portion (1g) of the extract was added to 1mL of 10% NaOH. Formation of a yellow coloration indicated the presence of flavonoids [10].

Sterols and terpens

They have been demonstrated by Liebermann-Burchard test [11].

Saponins

A portion (1g) of each extract was added to 5mL of distilled water and vigorously shaken for 2 min. Formation of froth indicated the presence of saponins [12].

Tannins

10 mL of distilled water was added to 2g of each extract, stirred and filtered. 1 mL of ferric chloride was then added to the filtrate. Formation of a blue-green precipitate indicated the presence of tannins [13].

Leuco-anthocyanins

0.5 mL of 12 N HCl was poured into 3 mL of extract. The acidified solution was brought to boiling water bath for 30 minutes. After cooling, the appearance of a purplish red color indicated the presence of leuco-anthocyanins [14].

Anthocyanins

To an infusion, 5 mL of 10% H_2SO_4 and 5ml of 50% NH_4OH were added. The appearance of a red color that turned purplish blue in basic medium indicates the presence of anthocyanins [15].

Quantitative Analysis of Phenolic Compounds.

Total polyphenols: The method of determination of total polyphenols consisted to use a mixture of phosphotungstic and phosphomolybdic acid which was reduced during the oxidation of phenols in the mixture of tungsten blue oxide and molybden [16, 17]. The absorbance was measured by a spectrophotometer (LANGE DR 3900) at 765 nm. Gallic acid was used as reference and the total polyphenol content in the extract was expressed in mg of gallic acid equivalents per gram of dry matter.

Total flavonoids

The method of aluminum trichloride ($AlCl_3$) was used to quantify the total flavonoids. This technique was based on the formation of the aluminum flavonoids complex that had a maximum absorption at 500 nm [16, 18].

Condensed tannins:

Condensed tannins dosing was achieved by using the method of vanillin sulfuric [10]. The principle of this assay was based on the binding of vanillin aldehyde group on the carbon in position 6 of the ring of the catechol to form a red coloured complex chromophore which absorbs at 510 nm.

Extraction of the essential oil

The dry leaves (100 g) of *Citrus sinensis* were separately ground and subjected to hydrodistillation with a Clevenger-type apparatus using 750 ml of deionised water for 4 h. The oil collected was dried over anhydrous sodium sulfate and stored at -20°C until using.

Chemical analysis of essential oil

Gas chromatography

GC analyses were performed on a Varian gas chromatograph, model CP-3380, with a flame ionization detector equipped with a silica capillary column: HP5 J&W Agilent (5%-phenyl-methylpolysiloxane) (30 m x 0.25 mm i.d. x 0.25 µm film); N_2 was the carrier gas at 0.8 mL/min; injection of 1 µL 1:10 CH_2Cl_2 solution, split ratio 1:100; injector temperature 220°C, detector temperature 250°C; temperature program 60-220°C at 3°C/min, then kept at 220°C during 20 min. The linear retention indices of the components were determined relative to the retention times of a series of n-alkanes with linear interpolation. The percentage composition of the essential oil was computed by the normalization method from the GC/FID peak areas on the HP5 capillary column, response factors being taken as one for all compounds.

Gas chromatography-mass spectrometry

GC/MS analyses were performed using a Hewlett–Packard GC 5890 series II equipped with a HP5 (5%-phenyl-methylpolysiloxane) fused silica column (30 m x0.25 mm; film thickness 0.25 µm) and a DB-Wax fused silica column (30 m x 0.25 mm; film thickness 0.25 µm) interfaced with a quadrupole detector (Model 5972) applying the same temperature program as for the GC/FID analyses with the apolar column; the temperature program was 70°C for 2 min, 70-220°C at 5°C/min, then kept at 220°C during 38 min using the polar column

(calculation of the linear retention indices on this column by coinjection with a series of n-alkanes); injector temperature, 220°C; MS transfer line temperature, 250°C; carrier gas, helium at a flow rate of 0.6 mL/min; injection type, split, 1:10 (1µL 10:100 CH_2Cl_2 solution); ionization voltage, 70 eV; electron multiplier 1460 eV; scan range 35-300 amu; scan rate, 2.96 scan/s. The identification of the constituents was based on comparison of their relative retention indices with either those of authentic samples or with published data in the literature [19] and by matching their mass spectra with those obtained with authentic samples and/or the NBS75K, Wiley 7th NIST 98 EPA/NIH, and FFNSC 2 libraries spectra.

Ethanolic, Hydroethanolic and aqueous Extracts' Preparation

50g of leaves powder of *C. sinensis* were mixed with 500cm^3 of concerned solvent. Ethanol 96°, water and ethanol-water (50/50) were used respectively for ethanolic extract aqueous extract and hydroethanolic extract. The mixture of leaves powder and solvent was filtered and evaporated to dryness at 40°C using rotary evaporator. Each residue was then allowed to cool, weighed and stored in refrigerator until needed.

Antibacterial Assay

Agar disc diffusion method

The agar disc diffusion method described by National Committee for Clinical Laboratory Standards reported by Mamadou et al. [20] was performed to determine the antibacterial activities of hydroethanolic, ethanolic, aqueous extracts and essential oil of *C. sinensis* against *E. coli* ATCC 25922, *S. aureus* 25923 and *K. pneumoniae* 818E, three pathogenic clinical bacteria of reference provided by the Laboratory of Bacteriology-Parasitology of Centre National Hospitalo-Universitaire (CNHU), the first and biggest hospital of Benin. The bacterial cultures were first grown on Nutrient agar plates at 37°C for 18 to 24 h. One or several colonies of the respective bacteria were transferred into normal saline and adjusted to 0.5 McFarland turbidity standards. The inoculum of the respective bacteria were streaked into Muller Hinton agar plates using a sterile swab and were then dried at 37°C during 15 min. Sterile filter discs having 6 mm of diameter soaked with 25 µL of 100 mg/mL of each non-volatile extract, 5 and 10 µL of pure essential oil extract separately were placed at the surface of Muller Hinton agar plates. The plates were incubated for 24 hrs at 37°C. The antibacterial activity was evaluated by measuring the clear zone surrounding the whatman paper. Standard discs of antibiotics (Gentamicin 10µg and Chloramphenicol 30µg) were applied as positive antibacterial controls. Each assay was performed in triplicates [21, 22].

Statistical Analysis

All assays were conducted at least three times with three different sample preparations. All data were expressed as mean ± standard deviation (SD). Analysis of variance was performed using In Stat (Graph Pad software, San Diego CA). A one-way ANOVA and unpaired Student's t-test were used for these analyses, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Chemical Composition of the Leaves Powder of *Citrus sinensis*.

The table 1 presents the results of chemical characterization of the leaves' extracts of *Citrus sinensis*. Various secondary metabolites have been identified in these extracts by a series of colorimetric reactions and precipitation specific to each class of active ingredients. Among these secondary metabolites, we have noticed saponins, gallics and catechic tannins, mucilages, flavonoids, anthocyanins, leuco-anthocyanins, sterols and terpenes. Only the aqueous extract contains sterols and terpenes but it didn't contain gallics tannins. The absence of alkaloids was observed in all extracts studied. Our results are similar to those obtained by Shalaby [23] who found in the leaves of the same plant coumarins, flavonoids and sterols. The same plant studied by Okwu et *al*. [24] revealed the presence of alkaloids, tannins, saponins and flavonoids. Phytochemical screening of whole plant of *Citrus sinensis* studied by Ekwenye and Edeha [25] revealed the presence of tannin, alkaloid, saponin, flavonoid, steroid and tripertenes.

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Table 1 : Main phytocompounds of *Citrus sinensis* non volatile extracts

Extracts / Secondary Metabolits		Aqueous extract	Hydroethanolic extract	Ethanolic extract
Alcaloïds		-	-	-
Anthocyanins		+	+	-
Leucoanthocyanins		+	+	+
Tanins	Gallic	-	+	+
	Catechic	+	+	+
Terpenes and Sterols		+	-	-
Flavonoïds		+	+	-
Saponins		+	+	+

Chemical Composition of the Essential oil hydrodistilled from Leaves of *C. sinensis.*

The chemical composition of the essential oil obtained from the leaves of *C. sinensis* is reported in table 2. Results showed that the major fraction of the oil is composed of hydrogenated monoterpens (74.0%) followed by (24.0%) of oxygenated monoterpens. As shown by their proportions given in table 2, the most abundant components in *Citrus sinensis* leaves' essential oil are sabinene (33.1%) and δ-3-carene (14.9%) while the minor components are limonene (8.8%), linalool (6.9%), (E)-β -ocimene (5.3%), myrcene (4.1%), terpinen-4-ol (3.4%), citronnellal (2.6%), terpinolene (2.1%), geranial (2.1%), β-pinene (1.7%), α-pinene (1.6%), citronellol (1.3%), γ-terpinene (1.2%) and neral (1.2%). The other minor compounds contents are less than 1.0% each one. These results are contrary to those obtained by Hamdan et *al*. [26] who found in the same leaf oil terpinen-4-ol (14.1%) as prominent compound. They are also contrary to those found (linalool 33.1%, terpineol 15.3%) and linalyl acetate 12.1%) as main components in the oil at Yucatan Peninsula by Pino et *al*. [27].

Table 2 : Chemical composition of the essential oil from *Citrus sinensis* leaves

N° Pic	KI	Nom des Composés	%
1	934	α-thujene	0.5
2	943	α-pinene	1.6
3	**986**	**Sabinene**	**33.1**
4	990	β-pinene	1.7
5	998	Myrcene	4.1
6	1014	α-phellandrene	0.7
7	**1021**	**δ-3-carene**	**14.9**
8	1025	α-terpinene	0.7
9	1033	p-cymene	0.9
10	**1038**	**Limonene**	**8.8**
11	1042	(Z)-β -ocimene	0.5
12	1054	(E)-β -ocimene	5.3
13	1066	γ-terpinene	1.2
14	1075	(Z)-sabinene hydrate	0.7
15	1097	Terpinolene	2.1
16	1107	Linalool	6.9
17	1158	Citronnellal	2.6
18	1175	Terpinen-4-ol	3.4
19	1188	α-terpineol	0.2
20	1232	citronellol	1.3
21	1248	Neral	1.2
22	1277	Geraniol	0.6
23	1279	Geranial	2.1
24	1357	Citronellyl acetate	1.2
25	1369	Neryl acetate	0.9
26	1382	geranyl acetate	0.8
27	1388	β-elemene	0.8
28	1420	(E)-β -caryophyllene	0.1
29	1455	α-humulene	0.1
30	1698	β-sinensal	0.4
31	1757	α-sinensal	0.1
Hydrogenated Monoterpens			**74.0**

Oxygenated Monoterpens		**24.0**
Hydrogenated Sesquiterpens		**1.0**
Oxygenated Sesquiterpens		**0.5**
Not identified		**0.1**
Total		**99.6**

Antibacterial Activity of *Citrus sinensis* Extracts

The results for antibacterial screening of extracts investigated are presented in Table 3. The non-volatile extracts were tested at a concentration of 100 mg.mL^{-1} in hydroethanolic solvent whereas 5µl and 10 µL per disc of essential oil of the plant were investigated. Tested at concentrations of 5µl and 10 µL, the essential oil had moderate activity on *S. aureus*. The oil presented also moderate activity on *E coli* strain at 10 µL and weak activity at 5µL. Strain of *K. pneumoniae* remained insensitive to varying oil concentrations tested. Globally, the non-volatile extracts of *C. sinensis* exhibit no antibacterial activity on the investigated strains. These results are similar to those obtained by Ekwenye and Edeha [25] who observed that crude leaves extracts of *C. sinensis* were not actives on strain of *E coli, S. aureus, K. pneumoniae* and *Pseudomonas aeruginosa.* In contrast, the essential oil of the plant had exhibited moderate activity on *S. aureus* and *E coli*. These results are in agreement with those of other researchers: 11.66±1.53mm on *S. aureus* and 8.0±0.00 mm on *E. coli* [28].

Table 3: Diameters (mm) of inhibition zone produced by the extracts on strains investigated

	Samples		Averages of the inhibition zone diameter		
			E. coli	*S. aureus*	*K. pneumoniae*
C. sinensis	Essential oil	5µL	08±00	**12±00**	07±01
		10µL	12±01	**14±00**	10±00
	Ethanolic extract		0.00	0.00	0.00
	Hydroethanolic extract		0.00	0.00	0.00
	Aqueous extrat		0.00	0.00	0.00
Gentamycine			23±1.5	29±00	25±00b
Chloramphénicol			30±00	32±00	28±00a
Ethanol/Eau (4/6)			0.00	0.00	0.00

Phenolic Compounds Contents of Ethanolic aqueous and Hydroethanolic Extracts

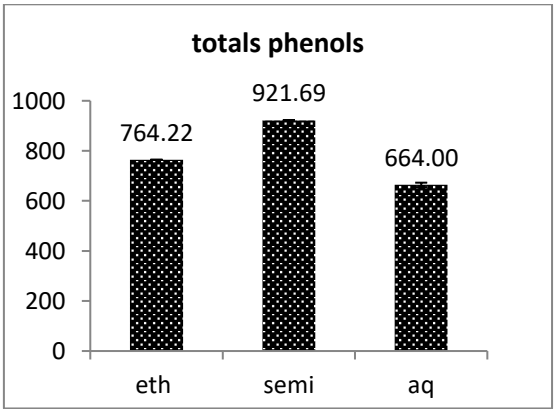

Figure 1: Content of polyphenols in ethanolic, hydroethanolic and aqueous extracts of leaves of *citrus sinensis* (mgEAG/gMS).

The contents of total polyphenols expressed out of equivalent mg of gallic acid per gram of dry matter (MS), flavonoids and condensed tanins expressed out of equivalent mg of catechin per gram (mgEC/g) are indicated on figures 1 to 3 and summarized in Table 4. These results showed that the extracts are rich in total phenol. Moreover, the ethanolic extract's content in flavonoids and condensed tannins were higher than those of aqueous and hydroethanolic extracts.

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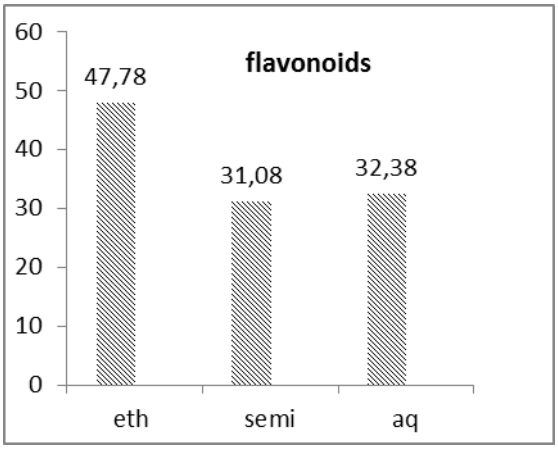

Figure 2: **Total flavonoids content of ethanolic, hydroethanolic and aqueous extracts of leaves of *citrus sinensis* (mgEC/gMS)**

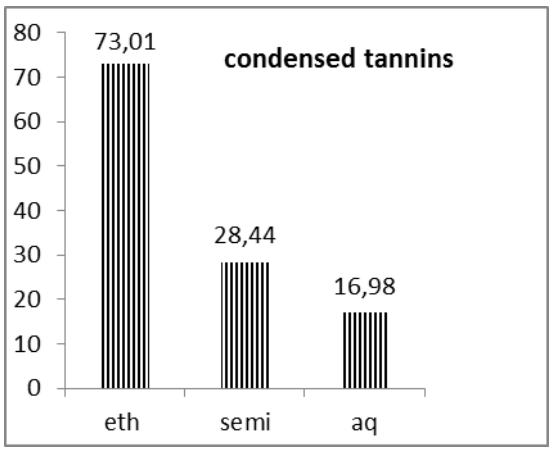

Figure 3: **Content of condensed tannins of ethanolic, hydroethanolic and aqueous extracts of *citrus sinensis*** (mgEC/gMS)

Table 4: Phenolic contents of extracts studied

Extracts	total Phenol (mgEAG/gMS)	Flavonoïds (mgEC/gMS)	Condensed tannins (mgEC/gMS)
Ethanolic extract	764.22±0.00	47.78±5.60	73.01±2.49
Hydroethanolic extract	921.69±0.35	31.08±0.11	28.44±0.41
Aqueous extract	664.00±8.80	32.38±0.25	16.98±0.95

Radical scavenging activity of extracts from the leaves of *Citrus sinensis*.

Table 5: Antiradical activity of *Citrus sinensis*' leaves extracts and references compounds

Extract/Compounds	IC_{50} (mg/mL)
Essential oil	6.01
Ethanolic extract	29.5
Hydroethanolic extract	35
Aqueous extrac	4.012
Acide gallique	0.03
quercétine	0.01
BHT	10

The results obtained for the antiradical activities of extracts are presented in the table 5. From the analysis of these results, it appears that the essential oil and aqueous extracts IC_{50} which are 6.01mg / mL and 4.02mg / mL respectively are more reactive than the BHT (IC_{50} = 10 mg / ml). They are less reactive than quercetin

(IC_{50} = 0.01 mg / mL) and gallic acid (IC_{50} = 0.03mg / ml). The antiradical activity of *Citrus sinensis* leaves oil obtained in our study is higher than those obtained by other researchers. In fact, Pierre and Singh found respectively IC_{50} equal to 7.4g/L [29] and 9.45µL/mL [6] in their studies.

CONCLUSION

The study highlighted the chemical composition of *Citrus sinensis* acclimated in Benin as well as antimicrobial and radical-scavenging properties of its extracts. The results showed that the essential oil of this plant is rich in monoterpens compounds dominated by sabinene. Non-volatile plant extracts contained many secondary metabolites that are mainly flavonoids, tannins, saponins and leucoanthocyanins. The aqueous extract and the oil of the plant possessed valuable antiradical properties than BHT. Of all the extracts tested, only the essential oil has been active on strains of *Staphylococcus aureus* and *Escherichia coli*. This oil could be used in the fight against certain infections caused by these sensitive bacteria.

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